Elucidating the impact of enhanced conversion of primary to secondary metabolism on phenylpropanoids secondary metabolites associated with flavor, aroma and health in tomato fruits

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ABSTRACT

- Targeted manipulating Phenylalanine (Phe) synthesis is one of the most powerful strategies to boost the biologically and economically important secondary metabolites, including phenylpropaniods, aromatic volatiles and specialized secondary metabolites.
- Over-expression of the petunia MYB transcript factor, ODORANT1 (ODO1), results in significant alterations of the levels of specific phenylpropanoid compounds in plants.
- Our previous studies indicated that ectopic expression of the feedback-insensitive AroG could break the bottleneck between primary and secondary metabolisms in tomato, thereby aiding in producing new tomato composition and identifying the unknown roles of multiple key regulators in specialized metabolism. Therefore, combining the AroG and ODO1 is of particular interest for elucidating the combined regulatory role of both of these genes in the Phe metabolic pathway, as well as generating tomato fruits that contain higher levels of secondary metabolites.
- Here, we performed the LC-MS and GC-MS analyses on fruits of four tomato genotypes, namely, wild type tomato fruits as well as tomato fruits expressing the AroG, ODO1 and the combination of AroG plus ODO1 (AO) genotypes. Our results elaborated that the levels of many of the Phe-derived metabolites were predominately altered in fruits of the AO genotype, compared to tomato fruits expressing either AroG or ODO1 individually. The levels of most of these metabolites were significantly stimulated, such as Tyrosine (Tyr), coumaric acid and ferulic acid derived metabolites, but the levels of some important secondary metabolites were reduced in the AO transgenic genotypes as compared to either AroG or ODO1 lines. Nevertheless, our results also revealed that the levels of aromatic volatiles were obviously down regulated in the AO, compared to that in AroG transgenic fruits, but were boosted while compared to the wild type and ODO1 transgenic fruits.
- Our results suggest that ODO1 expression may also have a negative effect on the production of some of the aromatic volatiles in tomato fruits, indicating that ODO1 acts as an important regulator of the shikimate pathway, which leads to the production of the aromatic amino acids and secondary metabolites derived from them.

Key words: AroG, ODO1, tomato, metabolism, shikimate pathway

Summary Sheet

PubType	IS only	Joint	US only
Review Article	1	0	0
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The Israeli group has over-expressed in tomato fruits a recombinant construct encoding a bacterial feedback-insensitive DAHP synthase, the first enzyme of the shikimate pathway.

The US group has over-expressed in tomato fruits the ODO1 gene encoding another downstream enzyme of the shikimate pathway.

The combination of these two enzymes together would have increased flux from primary metabolism towards the aromatic amino acids and hence led to increased levels of multiple secondary metabolites derived from the tomato fruits, with the aim of leading to improving the flavor and aroma of the tomato fruit without a negative effect on fruit shelf life.

ACHIEVEMENTS

- Targeted manipulating Phenylalanine (Phe) synthesis is one of the most powerful strategies to boost the biologically and economically important secondary metabolites, including phenylpropaniods, aromatic volatiles and specialized secondary metabolites.
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- Here, we performed the LC-MS and GC-MS analyses on fruits of four tomato genotypes, namely, wild type tomato fruits as well as tomato fruits expressing the AroG, ODO1 and the combination of AroG plus ODO1 (AO) genotypes. Our results elaborated that the levels of many of the Phe-derived metabolites were predominately altered in fruits of the AO genotype, compared to tomato fruits expressing either AroG or ODO1 individually. The levels of most of these metabolites were significantly stimulated, such as Tyrosine (Tyr), coumaric acid and ferulic acid derived metabolites, but the levels of some important secondary metabolites were reduced in the AO transgenic genotypes as compared to either AroG or ODO1 lines. Nevertheless, our results also revealed that the levels of aromatic volatiles were obviously down regulated in the AO, compared to that in AroG transgenic fruits, but were boosted while compared to the wild type and ODO1 transgenic fruits.
- Our results suggest that ODO1 expression may also have a negative effect on the production of some of the aromatic volatiles in tomato fruits, indicating that ODO1 acts as an important regulator of the shikimate pathway, which leads to the production of the aromatic amino acids and secondary metabolites derived from them.

There were no changes to the original collaboration

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Status	Type	Authors	Title	Journal	Volume: Pages	Year	Country
Published	Review Article	*Qingjun Xie1*, Simon Michaeli1,2*, Hadas Peled-Zehavi1, and Gad Galili1*	Chloroplast degradation: one organelle, multiple degradation pathways	***Trends in Plant Science***	20 : 265	***2015***	IS only
Accepted	Reviewed	*Vered Tzin1,*, Ilana Rogachev1, Sagit Meir1, Michal Moyal Ben Zvi2,†, Tania Masci2, Alexander Vainstein2, Asaph Aharoni1 and Gad Galili1,‡*	Tomato fruits expressing a bacterial feedback-insensitive 3-deoxy-d-arabino-heptulosonate 7-phosphate synthase of the shikimate pathway possess enhanced levels of multiple specialized metabolites and upgraded aroma	***Journal of Experimental Botany***	64 : 4441-4452	***2013***	IS only
Published	Reviewed	*Galili G, Avin-Wittenberg T, Angelovici R and A R I Fernie*	The role of photosynthesis and amino acid metabolism in the energy status during seed developmen	***Frontiers of Plant Science***	5 : 447	***2014***	IS only

INTRODUCTION

The Secondary metabolites produced in plants play multiple roles in the regulation of precursors for natural products, protection against ultraviolet (UV) light, pigments, signaling molecules, as well as cell wall components in plants (Tzin & Galili, 2010; Maeda & Dudareva, 2012; Liu *et al.*, 2015). As the most well characterized precursors for the synthesis of secondary metabolites, the aromatic amino acids (AAAs), including phenylalanine (Phe), Tryptophan (Trp) and Tyrosine (Tyr), are derived from Chorismate, the final metabolite of the shikimate pathway followed by the aromatic amino acids metabolic pathways. In fact, more than 30% of the fixed carbon in vascular plants is directed towards the synthesis of the aromatic amino acids via the shikimate and aromatic amino acid biosynthesis pathways, (Tzin & Galili, 2010; Maeda & Dudareva, 2012; Tohge *et al.*, 2013). So far, numerous genes and regulators of the AAA biosynthesis and the downstream secondary metabolic pathway have been extensively identified in various plants species. Among the interesting genes involved in the synthesis of the AAA-derived secondary metabolites are MYB transcription factors.

Many MYB transcription factors that participate in the biosynthesis of AAA have been isolated (Liu *et al.*, 2015). For example, the first gene in the shikimate pathway, termed the 3-deoxy-d-arabino-heptulosonate-7-phosphate synthase (DAHPS; EC 2.5.1.54), is regulated by the MYB transcription factor ATR1/MYB34 in *Arabidopsis* plants (Bender & Fink, 1998). Knockdown of *MYB8* in *Nicotiana attenuate* significantly causes the down-regulated expression of all seven shikimate pathway genes, eventually leading to a complete elimination of phenylpropanoid-polyamine conjugates (Kaur *et al.*, 2010). On the other hand, the role of MYB transcription factors in the Phe biosynthesis pathway has also been evident. For example, SIMYB12 is involved in the biosynthesis of phenylpropanoids, particularly flavonoids, in tomato (Adato *et al.*, 2009). The Phe-derived phenylpropanoids/benzenoids in petunia plants are controlled by a complex that contains a C2H2-type zinc finger DNA-binding protein, EPF1, and two R2R3-type MYB transcription factors, ODORANT1 (ODO1) and EMISSION OF BENZENOIDS II (EOBII) (Verdonk *et al.*, 2005; Spitzer-Rimon *et al.*, 2010; Van Moerkercke *et al.*, 2011). Recently, the R2R3-MYB-like gene, *EOBI*, has been implicated in the direct regulation of *ODO1* as well (Spitzer-Rimon *et al.*, 2012). Notably,

knockdown of *ODO1* in petunia results in higher accumulation of *EOBI* transcript level, compared to the control wild type petunia plants, suggesting a complex feedback loop between these regulatory factors. Interestingly, ectopic expression of *ODO1* in tomato fruits induced the levels of a specific subset of phenylpropanoid compounds but no changes were observed in the levels of Phe-derived flavor volatiles (Dal Cin *et al.*, 2011), implying that ODO1 may not participate in the biosynthesis of volatiles in tomato. However, this issue still requires further research.

To date, a few studies have provided various strategies to promote the production of Phe-derived secondary metabolites. One of the most interesting concerns regarding these issues is to focus on the manipulation of metabolic bottlenecks in the conversion of primary metabolism into secondary metabolism, particularly in the production of Phe-derived volatile and non-volatile secondary metabolites. Examples of enzymes associated with the synthesis of secondary metabolites include the aromatic L-amino acid decarboxylases (Gutensohn *et al.*, 2011), phenylacetaldehyde synthase (Kaminaga *et al.*, 2006) and isoeugenol synthase 1 (Dexter *et al.*, 2007). Our previous studies also indicated that overexpression of the bacterial AroG gene, encoding the DAHPS in plants, breaks a bottleneck in the conversion of primary metabolism via the aromatic amino acids into secondary metabolism, eventually leading to the enhanced levels of multiple specialized metabolites and upgraded aroma (Tzin *et al.*, 2012; Tzin *et al.*, 2013; Oliva *et al.*, 2015). These significant inductions of the synthesis of secondary metabolites also facilitate the discovery of novel fruit-specialized metabolites.

In the present research, we were specifically interested in: (i) elucidating the function of ODO1 in regulating the production of secondary metabolites, especially the Phe-derived volatiles and non-volatiles; (ii) produce tomato with higher favor secondary metabolites. Our results reveal that ODO1 mainly plays an antagonistic role with AroG enzyme in the regulation of synthesis of Phe-derived secondary metabolites, in particular the Phe-derived volatiles. In addition, ODO1 also coordinates the AroG to up-regulate some specialized secondary metabolites in tomato fruits. These results may shed new lights on understanding the multiple regulatory roles of MYB transcription factors in the conversion of primary to secondary metabolism.

MATERIALS AND METHODS

Plant material and growth conditions

All the plants used in this study were M82 background, including AroG and ODO1 transgenic plants. Generally, tomato seeds were sowed directly in the soil and grow in the greenhouse. Each biological repeat was a mixture of three to five individual fruits from the ripe red stage, and the peel and fleshy tissues (without the gel and seeds) were manually dissected and frozen in liquid nitrogen until use. To generate the co-expression lines of AroG and ODO1, the homozygous ODO1 transgenic plants were crossed with three independent homozygous AroG transgenic lines. Then the resulting F1 plants were used for this study.

LC-MS metabolomics analysis

Non-targeted metabolic analysis was performed using 100 mg of frozen powder from tomato skin and flesh tissues, extracted in 80% methanol. Samples were analyzed using an UPLCqTOF system (HDMS Synapt; Waters), with the UPLC C18 column connected online to a photodiode array detector and then to the MS detector, in MS^E acquisition mode. Sample preparation and injection conditions were performed as previously described (Mintz-Oron *et al.*, 2008). The analysis of the raw LC-MS (UPLC-qTOF-MS) data was performed using the XCMS software from the Bioconductor package (v. 2.1) for the R statistical language (v. 2.6.1) that performs chromatogram alignment, mass signal detection and peak integration (Smith *et al.*, 2006). XCMS was used with the following parameters: fwhm = 10.8, step = 0.05, steps = 4, mzdiff = 0.07, snthresh = 8, max = 1000. Injections of samples in the positive and negative ionization modes were performed in separate injection sets and pre-processing was done for each ionization mode independently. The list of putatively identified compounds (totally 69 metabolites in both skin and flesh), including their exact masses, retention times and the main fragments are present in Supplemental Table S1. Differential mass ions were determined using a Student's t-test (JMP software) and 17 differential metabolites were subsequently assigned. Principal Component Analysis

(PCA) plot was performed by the T-MEV4 software (Scholz *et al.*, 2004). A Student's t-test analysis was performed on metabolites level using the JMP software (SAS).

RESULTS

Generation of hybrid tomato lines overexpressing the AroG and ODO1 proteins

Since the AroG and ODO1 have been known to regulate the Secondary metabolisms in tomato (Dal Cin *et al.*, 2011; Tzin *et al.*, 2012), it was intriguing to co-express these two genes together and thereby test their effects on the levels of the primary and secondary metabolites in tomato fruits. To this end, the ODO1-overexpressing tomato line 8117 was selected for the following studies due to its highest expression in all the transgenic tomato lines (Dal Cin *et al.*, 2011). Subsequently, three independent AroG-overexpressing tomato lines were individually crossed with the ODO1-8117 line to generate the transgenic tomato lines whose fruits co-express the AroG and ODO1 transgenes (hereafter refers to as AO). Quantitative real time PCR (qRT-PCR) analysis revealed that the transcript levels of *ODO1* was similar between the *ODO1* transgenic plant and most of the AO lines (Figure 1A), while the expression of AroG was attenuated in most of the AO lines, compared to their corresponding AroG parent lines (Figure 1B). These results imply that ODO1 may inhibit the expression of AroG.

Since the expression of AroG in the AO69 (AroG69 × ODO1) lines was slightly down regulated rather than that in other AO lines when compared to their corresponding AroG transgenic lines, we thus selected the AO69 for the following studies. In consistence to previous reports (Dal Cin *et al.*, 2011; Tzin *et al.*, 2012), phenotypic analysis showed that overexpressing ODO1 retarded the maturation and/or ripening of the tomato fruits, while overexpression of AroG had no influence on the fruit shelf life. Notably, the AO fruit displayed the similar phenotype as that of ODO1 transgenic lines (Figure 1C), further suggesting that ODO1 either directly represses AroG or functions downstream of AroG.

Metabolic changes among the wild type, ODO1, AroG and AO fruits

To explore the effect of AO on the metabolism of the tomato fruit, two types of tissues (skin and flesh) from the ripen stage were separately collected for the LC-MS analysis. The PCA analysis showed that the metabolic profiles of the wild type (M82 cultivar), AroG, ODO1 and AO genotypes were completely separated from each other in both the skin and flesh tissues, respectively. To further test whether AO altered the levels of metabolites related to the shikimate pathway, we focused on related metabolites that could be detected by the LC-MS platform. Our results showed that the levels of three AAAs were significantly altered among the four genotypes. As shown in the Fig. 2C and 2D, the levels of Phe and Trp were down-regulated and up-regulated in the ODO1 and AroG lines, respectively, compared to the wild type; the level of Phe was increased in the AO genotype, compared to both wild type and ODO1 genotypes, but slightly decreased when compared to the AroG genotype, while the contents of Trp were increased in the AO compared to the ODO1 genotypes, but slightly decreased when compared to both the wild type and AroG genotypes. These results suggest that ODO1 apparently plays a negative role in the biosynthesis of Phe and Trp. Notably, the levels of Tyr were similar between the wild type and ODO1 genotypes, but up regulated in the AroG and AO genotype (Figure 2 and Table 1). The highest levels of Tyr were present in the AO tomato fruits, suggesting that both ODO1 and AroG positively regulate the accumulation of Tyr.

Co-expression of both AroG and ODO1 alters the profiles of Phe-derived non-volatile metabolites in the tomato fruits

We were particularly interested in examining the changes of Phe-derived secondary metabolites, particularly the phenylpropanoids. Therefore, we first investigated the metabolite changes in the skins of the WT, AroG, ODO1 and AO tomato fruits (Supplemental Table S1). Our results showed that the level of eight and 12 metabolites in ODO1 was significantly up regulated or down regulated as compared to that in wild type (Table 1). Notably, these down-regulated metabolites mainly included narigenin derived

metabolites. Regarding the AroG, there were 10 metabolites significantly up-regulated as compared to wild type, which mainly included Phe, Tyr, caffeic acid, coumaric acid derived metabolites. On the other hand, only two metabolites, methy-butanol-hexose-pentose and phloretin-trihexose, were down regulated in AroG as compared to those in wild type (Table 1). In total, the level of 20 and 11 metabolites in AO was up regulated or down regulated as compared to that in wild type. These up-regulated metabolites included ferulic acid, kaempferol and quercetin derived metabolites. Among them, the level of eight metabolites were significantly up regulated in AO as compared to those in either the wild type, ODO1 or AroG genotypes (Table 1). Interestingly, we found that the variation pattern of metabolites in ODO1 displayed similar trends in AO as compared to wild type. For example, the upregulation of ferulic acid and kaempferol derived metabolites in ODO1 as compared to wild type was also detected in AO, whereas the downregulation of naringenin and quercetin derived metabolites in ODO1 was found in AO as well. These results suggest that ODO1 plays a key role in regulating these secondary metabolites. In addition, we also found that a certain part of metabolites in AO, which has no significant change in ODO1 as compared to wild type, showed similar trends as those in AroG while compared to wild type.

In terms of the flesh tissues of the four genotypes, our observations revealed that 40 metabolites were identified in total. Of these metabolites, the levels of 15 metabolites were differentially expressed among wild type, ODO1, AroG and AO lines (Table 2). The levels of the rest metabolites were not significantly altered among the four genotypes (Supplemental Table S1). Our results indicated that three and one metabolites up or down regulated in ODO1 as compared to wild type, whereas only one metabolite in AroG was significantly upregulated as compared to wild type (Table 2). Surprisingly, 14 out of the 15 differentially expressed metabolites exhibited upregulation pattern in AO as compared to wild type. These metabolites mainly included caffeic acid, coumaric acid and feruoyl derived metabolites. Furthermore, our results also showed that the nine out of the 14 metabolites was significantly up regulated in AO as compared to either wild type, ODO1 or AroG. These results indicate that combined ODO1 and AroG significantly benefit the breakdown of the bottleneck of Phe-derived metabolites in either ODO1 or AroG in tomato flesh. Integrating the results of the skin and flesh analyses, we concluded that co-expression of both the ODO1 and AroG transgenes promotes the accumulation of coumaric acid,

caffeic acid and ferulic acid derived metabolites and the ODO1 gene apparently serves as a negative regulator of the biosynthesis of naringenin and quercetin derived secondary metabolites.

Co-expression of both AroG and ODO1 alters the profiles of multiple volatile metabolites

To further explore the metabolites produced in the AO fruits, compared to the fruits of the other tomato genotypes, we also performed a GC-MS analysis on the wild type, ODO1, AroG and AO fruits. The PCA analysis indicated that the metabolic profiles of the four genotypes were significantly separated (Figure 3). To further elucidate whether the levels of metabolites associated with the shikimate and AAA biosynthesis pathways were altered in the AO expressing fruits, compared to the ODO1 and AroG expressing fruits, we focused on related metabolites that could be detected by the GC-MS platform. Our results indicated that the levels of the Phe-derived volatiles were reduced in the AO, compared to that in the AroG genotypes, but increased when compared to both the wild type and ODO1 genotypes (Figure 4). In addition, the levels of other volatile phenylpropanoids in the AO tomato fruits were also significantly decreased, compared to the fruits expressing the single AroG transgene (Figure 5). The reason for the reduction in the levels of the various Phe-derived volatiles in the AO, compared to the AroG tomato fruits is still not entirely clear. It is thus possible that much of the effects of ODO1 expression in term of accumulation of secondary metabolites are due to its turning on PAL and other phenylpropanoid synthesis genes. This likely causes an overall increased levels of a lot more downstream phenylpropanoids in the ODO1 expressing plants, compared to other volatile compounds and even much more in the AO lines.

Interestingly, an enhancement of branch-chain amino acid (BCAA)-derived volatiles was detected in the AO, compared to that of AroG genotypes but had no significant changes when compared to that of ODO1 genotype (Figure 6). This implies a positive effect of ODO1 in the AroG-mediated biosynthesis of BCAA-derived volatiles. Notably, the levels of BCAA-derived volatiles were generally lower in the transgenic fruits than that in wild type

fruits, exclusively 2-methyl butanal (Figure 6). Taken together, our results suggest that ODO1 negatively regulates the biosynthesis of volatiles.

DISCUSSION

Secondary metabolites produced in tomato fruits, such as the phenylalanine (Phe) derived metabolites and volatile compounds, play important roles in the tomato fruit flavor (Klee & Giovannoni, 2011), as well as plant defense mechanisms (Dudareva *et al.*, 2004). Most volatile compounds accumulating in the ripe tomato fruits were derived from Phe. Therefore, genetic manipulation of Phe-derived metabolic pathways has become a powerful strategy for tomato breeding. Our results suggest that co-expression of both AroG and ODO1 enables the generation of new tomato composition, in particular the Phe-derived metabolites.

Complicated regulatory role of ODO1 in secondary metabolism

The secondary metabolites derived from the aromatic amino acids (AAAs), have been well characterized, which include Phe, Tryptophan (Trp) and Tyrosine (Tyr). These three aromatic amino acids are derived from the final step of the shikimate pathway (Tzin & Galili, 2010; Maeda & Dudareva, 2012; Tohge *et al.*, 2013), and their biosynthesis have been reported to be regulated by MYB transcription factors in the plant kingdom (Liu *et al.*, 2015). Examples of these studies include ATR1/MYB34 in Arabidopsis (Bender & Fink, 1998), MYB8 in *Nicotianna attenuate* (Kaur *et al.*, 2010) and SlMYB12 in tomato (Adato *et al.*, 2009). Recently, increasing evidences have implicated the importance of another MYB gene, ODORANT1 (ODO1), in regulating the metabolism of Phe-derived phenylpropanoids and benzenoids in petunia (Verdonk *et al.*, 2005; Spitzer-Rimon *et al.*, 2010; Van Moerkercke *et al.*, 2011). Notably, ectopic over-expression of ODO1 in tomato promotes the levels of phenylpropanoid specialized metabolites, but has no effect on that of the Phe-derived flavor volatiles (Dal Cin et al., 2011). However, our results indicated that ODO1 plays a negative role in the accumulation of Phe-derived volatiles and other phenylpropanoid volatiles, but positively regulates that of BCAA-derived volatiles. A previous study also indicated that ODO1 induces an up-regulation of a subset of specialized

secondary metabolites, particularly ferulic acid, but attenuates the levels of other phenylpropanoids, such as naringenin. Moreover, the levels of caffeic acid, coumaric acid and quercetin were almost equal between the wild type and ODO1 transgenic plants (Dal Cin *et al.*, 2011). In the AroG and ODO1co-expression line, our results clearly indicated that the biosynthesis or the metabolic flux into ferulic acid, caffeic acid, coumaric acid and kaempferol derived metabolites was increased, as well as that of two naringenin and one quercetin derived metabolites in the skin tissues. Similar changes were also found in the fleshy tissue of the AroG plus ODO1co-expression line. These results suggest that ODO1 is also involved in the regulation of other phenylpropanoids derived metabolites in addition to the previously discovered metabolites.

AroG expressing transgenic tomato plants are candidate recipient parents for producing favorable varieties and may also promote the discovery of the regulatory role of interesting genes in primary and secondary metabolism

Our previous studies have implicated the multiple contributions of AroG to broad primary and secondary metabolites in various plants (Tzin *et al.*, 2012; Tzin *et al.*, 2013; Oliva *et al.*, 2015). Using these AroG expressing lines as recipient parents, specifically targeted manipulation of interesting genes or enzymes in the shikimate pathway allows the generation of new secondary metabolites that may lead to new plant varieties. Here, we produced the co-expression line of AroG plus ODO1, in which a series of interesting secondary metabolites were significantly altered. For example, the antioxidant metabolites (e. g. coumaric acids and ferulic acids) have been implicated the importance for the plant defense and human health (Korkina, 2007). Our results demonstrated that the combination of AroG and ODO1 largely boosts the accumulation of these metabolites, compared to that of expression of either a single AroG or a single ODO1. Interestingly, Phe and Typ, two precursors of the general phenylpropanoid pathway, showed opposite trends between the AroG and ODO1 expression plants, eventually leading to the modest changes of these two metabolites in the co-expression line of AroG and ODO1. On the other hands, another precursor of phenylpropanoid pathway, namely Tyr, was induced in both AroG and ODO1 plants, resulting in largely boosting of Tyr in the co-expression line containing both genes. Tomatine has been reported to be beneficial for human diet and health, such as

Anticarcinogenic effects (Friedman, 2002; Friedman, 2013). Although down regulation of tomatine was found in the single AroG plant, co-expression of AroG and ODO1 increased the abundance of tomatine since the ODO1 has a positive effect on the accumulation of this metabolite. Taken together, selective integration of AroG and ODO1 in tomato significantly alters the secondary metabolism, eventually generating large-scale of interesting metabolites (Figure 7).

Overall, co-expressing AroG and ODO1 in tomato promotes the contents of specialized secondary metabolites, but retards that of volatiles compounds, suggesting that a combination of AroG and key regulators (and/or enzymes) from shikimate pathway may enable the generation of new tomato varieties that contain large-scales of favorable secondary metabolites. In addition, our results also indicate that AroG-mediated boosting of secondary metabolites enables the discovery of unknown regulatory roles of ODO1, or even other MYB transcription factors.

TABLE 1. Differentially expressed metabolites in the fruits skin of the four genotypes.

metabolite	WT	ODO1	AroG	AO
5-Caffeoylquinic acid	1 ± 0.03 (ab)	0.57 ± 0.13 (a)	1.43 ± 0.5 (b)	0.74 ± 0.22 (ab)
Benzyl alcohol-dihexose	1 ± 0.39 (a)	0.45 ± 0.39 (a)	5.55 ± 4.41 (b)	3.02 ± 1.2 (ab)
Benzyl alcohol-hexose-pentose	1 ± 0.24 (a)	0.54 ± 0.13 (a)	3.34 ± 1 (b)	3.32 ± 0.54 (b)
Caffeic acid hexose Isomer 1	1 ± 0.06 (a)	0.59 ± 0.09 (a)	60.25 ± 33.12 (b)	7.07 ± 3.03 (a)
Caffeic acid-hexose Isomer 2	1 ± 0.03 (ab)	0.69 ± 0.21 (a)	6.74 ± 4.43 (b)	1.32 ± 0.21 (ab)
Caffeic acid-hexose Isomer 3	1 ± 0.09 (a)	0.47 ± 0.07 (a)	1.19 ± 0.61 (a)	**4.06 ± 0.24 (b)**
Chlorogenic acid	1 ± 0.11 (a)	0.53 ± 0.22 (a)	2.48 ± 0.93 (b)	0.86 ± 0.28 (a)
Coumaric acid-hexose Isomer 1	1 ± 0.71 (a)	0.4 ± 0.16 (a)	102.99 ± 72.25 (b)	59.7 ± 11.02 (ab)
Coumaric acid-hexose Isomer 2	1 ± 0.26 (a)	0.57 ± 0.06 (a)	8.68 ± 4.74 (b)	7.22 ± 2.38 (ab)
Coumaric acid-hexose Isomer 3	1 ± 0.16 (a)	22.73 ± 2.87 (a)	3.42 ± 1.48 (a)	88.03 ± 18.03 (b)
Coumaroylquinic acid	1 ± 0.12 (a)	0.69 ± 0.18 (a)	11.64 ± 5.29 (b)	3.19 ± 0.93 (a)
di hydroxy-methyl-benzoic acid hexose	1 ± 0.21 (a)	3.57 ± 0.9 (b)	0.62 ± 0.06 (a)	2.93 ± 0.27 (b)
Ferulic acid hexose Isomer 2	1 ± 0.12 (a)	6.35 ± 2.94 (a)	0.42 ± 0.07 (a)	**23.31 ± 9.13 (b)**
Ferulic acid-hexose Isomer 1	1 ± 0.56 (a)	3.72 ± 1.96 (a)	1.85 ± 0.96 (a)	**18.61 ± 3.31 (b)**
Feruloyl quinic acid	1 ± 0.17 (a)	24.71 ± 6.29 (b)	1.63 ± 0.42 (a)	21.65 ± 6.04 (b)
Feruloyltyramine	1 ± 0.49 (a)	7.92 ± 2.5 (a)	3.07 ± 1.74 (a)	**21.84 ± 7.26 (b)**
Feruoylquinic acid-O-hexoside	1 ± 0.32 (a)	38.31 ± 12.84 (b)	3.06 ± 3.62 (a)	44.48 ± 11.63 (b)
Hydrocinnamic acid-hexose	1 ± 0.25 (a)	0.26 ± 0.05 (a)	3.41 ± 1.65 (b)	4.02 ± 0.77 (b)
Hydroxybenzoic acid-hexose	1 ± 0.09 (b)	0.13 ± 0.02 (a)	1.29 ± 0.28 (b)	0.29 ± 0.09 (a)
Hydroxylated naringenin (Eriodictyol) (S)	1 ± 0.07 (b)	0.15 ± 0.05 (a)	0.7 ± 0.63 (ab)	0.28 ± 0.12 (ab)
Hydroxylated naringenin chalcone	1 ± 0.05 (b)	0.1 ± 0.01 (a)	0.56 ± 0.49 (ab)	0.07 ± 0.02 (a)
Hydroxy-Lycoperoside A;Hydroxy-Lycoperoside B or Hydroxy-Lycoperoside C	1 ± 0.09 (ab)	1.13 ± 0.31 (b)	0.45 ± 0.15 (a)	0.97 ± 0.31 (ab)
Kaempferol hexose-deoxyhexose-hexose	1 ± 0.32 (a)	2.41 ± 0.29 (b)	0.98 ± 0.33 (a)	2.27 ± 0.43 (b)
Kaempferol-3-O-feruloyl triglucoside-7-O-glucoside	1 ± 0.71 (a)	62.06 ± 16.48 (b)	1.7 ± 0.81 (a)	63.52 ± 10.84 (b)
kaempferol-3-O-p-coumaroyl triglucoside-7-O-glucoside	1 ± 0.2 (a)	55.5 ± 10.67 (b)	4.82 ± 2.74 (a)	94.11 ± 34.44 (b)
Kaempferol-glucose-rhamnose	1 ± 0.23 (b)	0.33 ± 0.11 (a)	0.47 ± 0.37 (ab)	0.21 ± 0.11 (a)
Kaempferol-hexose-deoxyhexose-pentose	1 ± 0.1 (b)	0.28 ± 0.02 (a)	0.64 ± 0.45 (ab)	0.37 ± 0.05 (a)
Lycoperoside A/B or Lycoperoside C	1 ± 0.07 (a)	1.87 ± 0.39 (b)	0.62 ± 0.29 (a)	0.82 ± 0.37 (a)
Methyl-butanol-hexose-pentose	1 ± 0.06 (b)	1.5 ± 0.31 (b)	0.17 ± 0.03 (a)	**2.74 ± 0.3 (c)**
Naringenin	1 ± 0.21 (b)	0.1 ± 0.02 (a)	0.62 ± 0.45 (ab)	0.08 ± 0.05 (a)
Naringenin chalcone-dihexose	1 ± 0.5 (a)	1.16 ± 0.17 (a)	5.61 ± 3.87 (ab)	9.39 ± 1.06 (b)
Naringenin chalcone-hexose Isomer 2	1 ± 0.14 (b)	0.17 ± 0.02 (a)	0.77 ± 0.58 (ab)	0.28 ± 0.16 (ab)
Naringenin hexose or Naringenin	1 ± 0.2 (ab)	0.29 ± 0.02 (a)	0.75 ± 0.45 (ab)	1.5 ± 0.45 (b)

chalcone hexose				
Naringenin-dihexose Isomer 1	1 ± 0.24 (b)	0.26 ± 0.11 (a)	0.83 ± 0.42 (ab)	0.21 ± 0.11 (a)
Naringenin-dihexose Isomer 2	1 ± 0.12 (a)	1.61 ± 0.06 (a)	1.34 ± 0.89 (a)	**3.31 ± 0.45 (b)**
Phenylalanine	1 ± 0.18 (a)	0.41 ± 0.05 (a)	34.58 ± 13.13 (b)	17.93 ± 4.63 (ab)
Phloretin-di-C-hexose	1 ± 0.08 (b)	0.18 ± 0.05 (a)	0.68 ± 0.5 (ab)	0.19 ± 0.06 (a)
Phloretin-trihexose	1 ± 0.08 (b)	0.11 ± 0.03 (a)	0.36 ± 0.26 (a)	0.18 ± 0.05 (a)
Quercetin hexose-hexose	1 ± 0.21 (a)	0.75 ± 0.07 (a)	1.63 ± 0.82 (a)	**3.53 ± 0.97 (b)**
Quercetin-dihexose-deoxyhexose	1 ± 0.2 (a)	2.11 ± 0.24 (c)	1.18 ± 0.42 (ab)	2.06 ± 0.46 (bc)
Quercetin-dihexose-deoxyhexose-p-coumaric acid	1 ± 0.07 (a)	4.9 ± 0.86 (ab)	1.98 ± 0.94 (ab)	5.56 ± 2.91 (b)
Quercetin-hexose-deoxyhexose-pentose	1 ± 0.03 (b)	0.24 ± 0.02 (a)	0.58 ± 0.37 (ab)	0.14 ± 0.06 (a)
Quercetin-hexose-deoxyhexose-pentose-p-coumaric acid	1 ± 0.07 (ab)	0.1 ± 0.02 (a)	4.33 ± 2.57 (b)	0.38 ± 0.15 (a)
Quercetin-O-dihexose-O-deoxyhexose	1 ± 0.1 (b)	0.32 ± 0.02 (a)	0.71 ± 0.2 (b)	0.27 ± 0.13 (a)
Tomatine (S)	1 ± 0.09 (ab)	1.31 ± 0.26 (b)	0.48 ± 0.17 (a)	0.74 ± 0.3 (ab)
Tricaffeoylquinic acid	1 ± 0.13 (bc)	0.1 ± 0.02 (ab)	1.09 ± 0.69 (c)	0.07 ± 0.05 (a)
Tryptophan	1 ± 0.09 (ab)	0.28 ± 0.11 (a)	1.28 ± 0.58 (b)	0.83 ± 0.06 (ab)
Tyrosine	1 ± 0.14 (a)	1.25 ± 0.23 (a)	13.71 ± 3.22 (b)	**54.64 ± 5.56 (c)**

Numbers (n = 3; mean ± standard error) are the fold change as compared to wild type, and the numbers in bold indicate significant up-regulation of the corresponding metabolites in AO, as compared to that in the other three genotypes. Boxes in yellow or green represent the upregulation or downregulation of the level of metabolite in corresponding genotype as compared to that in wild type. The letters in baskets represent significant difference among the four genotypes by using ANOVA ($p < 0.05$) and the Tukey-test for corrections for multiple comparisons ($p < 0.05$).

TABLE 2. Differentially expressed metabolites in the flesh of AO fruits.

Metabolites	WT	ODO1	AorG	AO
Caffeic acid-hexose Isomer 1	1 ± 0.36 (a)	0.43 ± 0.09 (a)	17.81 ± 11.61 (ab)	27.56 ± 9.07 (b)
Caffeic acid-hexose Isomer 2	1 ± 0.09 (a)	1.17 ± 0.68 (a)	5.24 ± 4.06 (ab)	8.63 ± 2.52 (b)
Caffeic acid-hexose Isomer 3	1 ± 0.09 (a)	1.78 ± 0.44 (a)	0.73 ± 0.56 (a)	**8.17 ± 0.66 (b)**
Coumaric acid hexose Isomer 1	1 ± 0.3 (a)	0.87 ± 0.37 (a)	2.1 ± 1.6 (a)	**8.1 ± 1.66 (b)**
Coumaric acid hexose Isomer 2	1 ± 0.14 (a)	0.99 ± 0.27 (a)	1.25 ± 0.88 (a)	**9.08 ± 2.18 (b)**
Coumaric acid hexose Isomer 3	1 ± 0.54 (a)	5.56 ± 1.94 (a)	4.85 ± 3.03 (a)	**31.06 ± 7.15 (b)**
Coumaroylquinic acid	1 ± 0.3 (ab)	0.77 ± 0.18 (a)	1.24 ± 0.94 (ab)	5.28 ± 1.21 (b)
Ferulic acid-hexose Isomer 1	1 ± 0.07 (a)	0.81 ± 0.26 (a)	0.85 ± 0.57 (a)	**3.77 ± 2.02 (b)**
Feruloyl quinic acid	1 ± 0.25 (a)	5.26 ± 0.7 (bc)	2.01 ± 1.46 (ab)	**13.41 ± 2.31 (c)**
Feruoylquinic acid-O-hexoside	1 ± 0.23 (a)	14.88 ± 7.7 (b)	0.83 ± 0.43 (a)	**27.66 ± 3.8 (c)**
Hydrocinnamic acid-hexose	1 ± 0.25 (a)	3.77 ± 1.11 (a)	2.21 ± 1.93 (a)	**32.22 ± 2.86 (b)**
Methyl-butanol-hexose-pentose	1 ± 0.45 (a)	10.9 ± 1.53 (b)	0.18 ± 0.15 (a)	4.23 ± 1.48 (c)
Naringenin chalcone	1 ± 0.31 (b)	0.06 ± 0.01 (a)	0.44 ± 0.32 (ab)	0.07 ± 0.04 (a)
Phenylalanine	1 ± 0.25 (ab)	0.07 ± 0.01 (a)	4.66 ± 3.36 (b)	2.77 ± 0.67 (ab)
Tyrosine	1 ± 0.1 (a)	0.82 ± 0.14 (a)	7.27 ± 3.48 (b)	**21.33 ± 2.79 (c)**

Numbers (n = 3; mean ± standard error) are the fold change as compared to wild type, and the numbers in bold indicate significant up-regulation of the corresponding metabolites in AO, as compared to that in the other three genotypes. Boxes in yellow or green represent the upregulation or downregulation of the level of metabolite in corresponding genotype as compared to that in wild type. The letters in baskets represent significant difference among the four genotypes by using ANOVA ($p < 0.05$) and the Tukey-test for corrections for multiple comparisons ($p < 0.05$).

FIGURE LEGENDS

Figure 1. Characterization of the tomato line co-expressing AroG and ODO1.

A&B, The copy number of the ODO1 (A) and AroG (B) genes in fruits of the four tomato genotypes, namely, the wild type M82, ODO1, AroG, AroG x ODO1. (C) Phenotype of the co-expression line of the AroG and ODO1 in the fruit.

Figure 2. Metabolic profiles of the four tomato genotypes determined by GC-MS.

PCA analyses of the metabolites identified in peel (A) and flesh (B) tissues. C to E, represents the fold changes in the levels of Phe (C), Trp (D) and Tyr (E) in the four genotypes.

Figure 3. The levels of Phe-derived volatiles in the four tomato genotypes. A to D, the levels of 2-phenylethanol (A), Phenylacetaldehyde (B), 2-Phenylacetonitrile (C) and 1-nitro-2-phenylethane (D) in the four tomato genotypes.

Figure 4. The levels of volatile phenylpropanoids in the four tomato genotypes. A to C, The levels of eugenol (A), methylsalicylate (B) and guaiacol (C) in the four tomato genotypes.

Figure 5. The levels of BCAA-derived volatiles in the four tomato genotypes. A to D, the levels of 2-methyl butanal (A), 3-methyl butanal (B), 2-methyl-1-butanol (C) and 3-methyl-1-butanol (D) in the four tomato genotypes.

Figure 6. Schematic representation of the changes of the primary and secondary metabolites in the four tomato genotypes. Green boxes indicates the down-regulation of the given metabolites, while red boxes indicate the up-regulation of the given metabolites. Boxes with dash lines represent no corresponding metabolites detected in the fleshy tissue.

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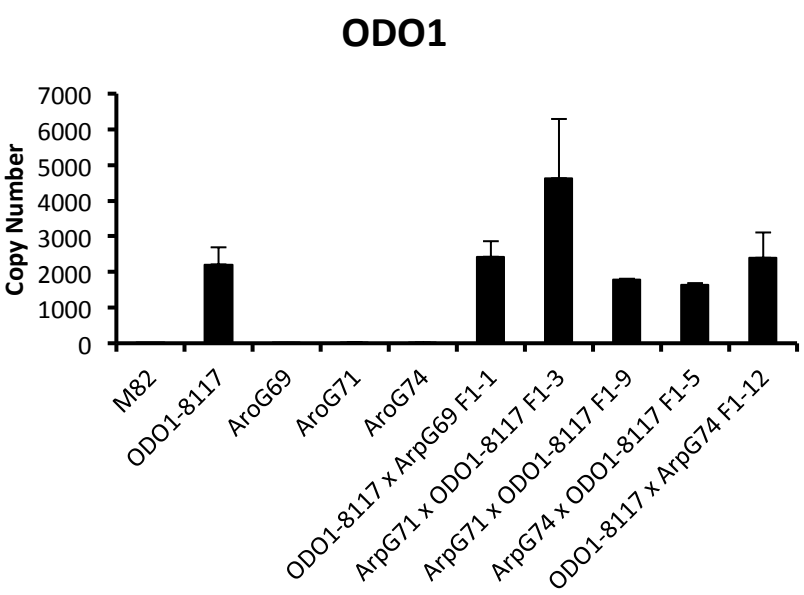
Fig. 1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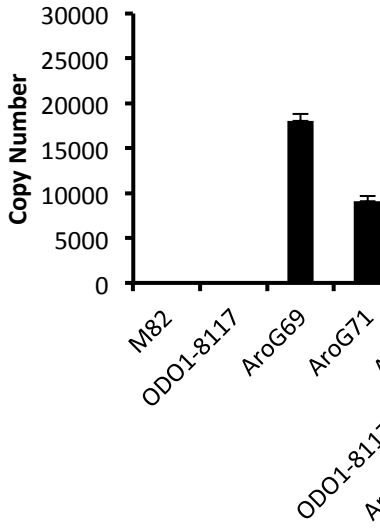
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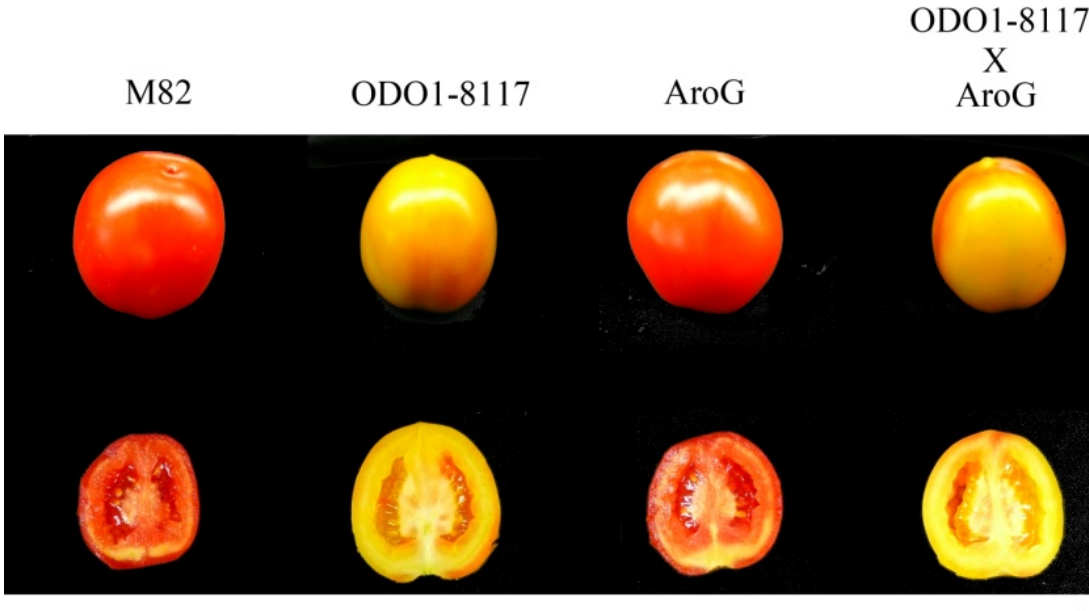
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Fig. 2

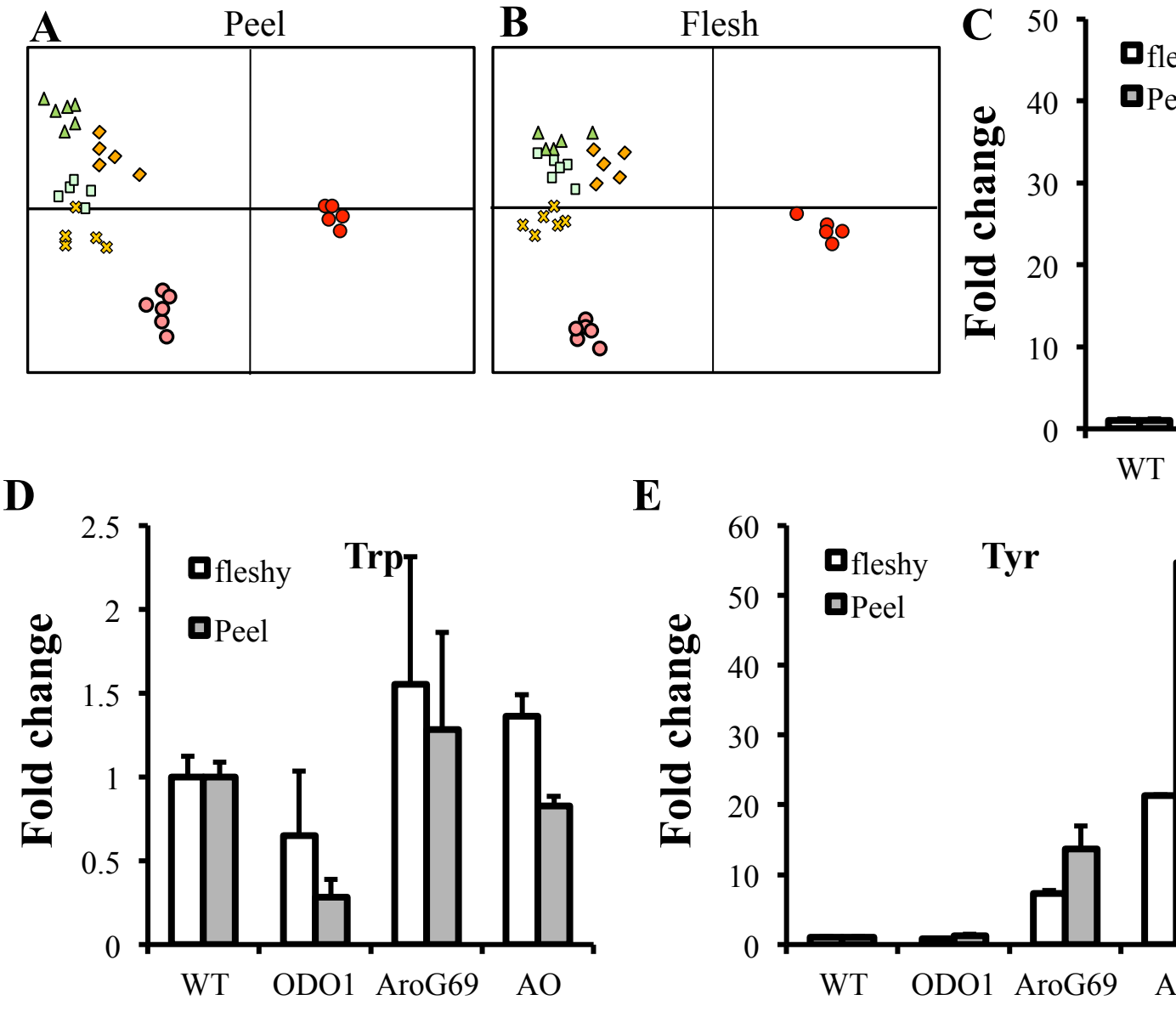
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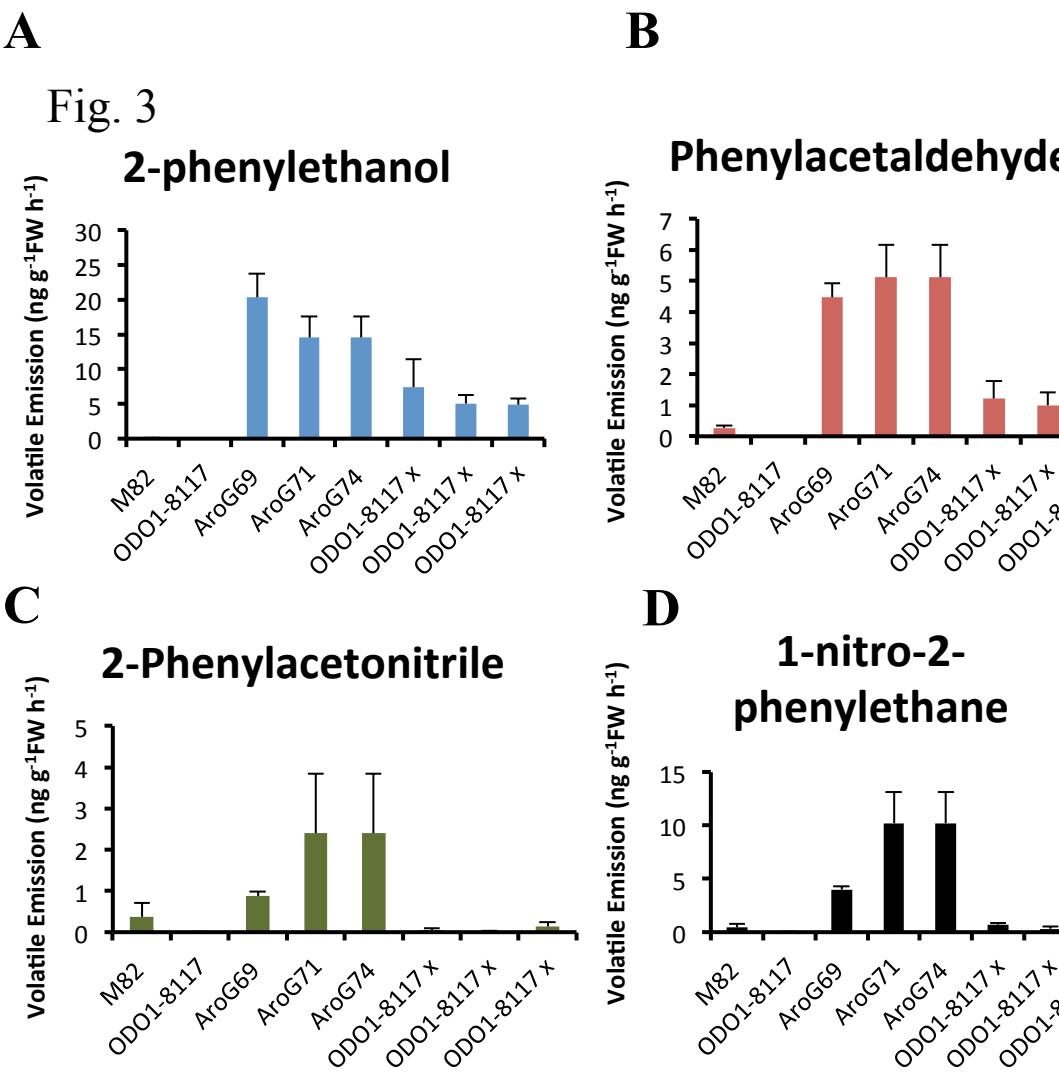
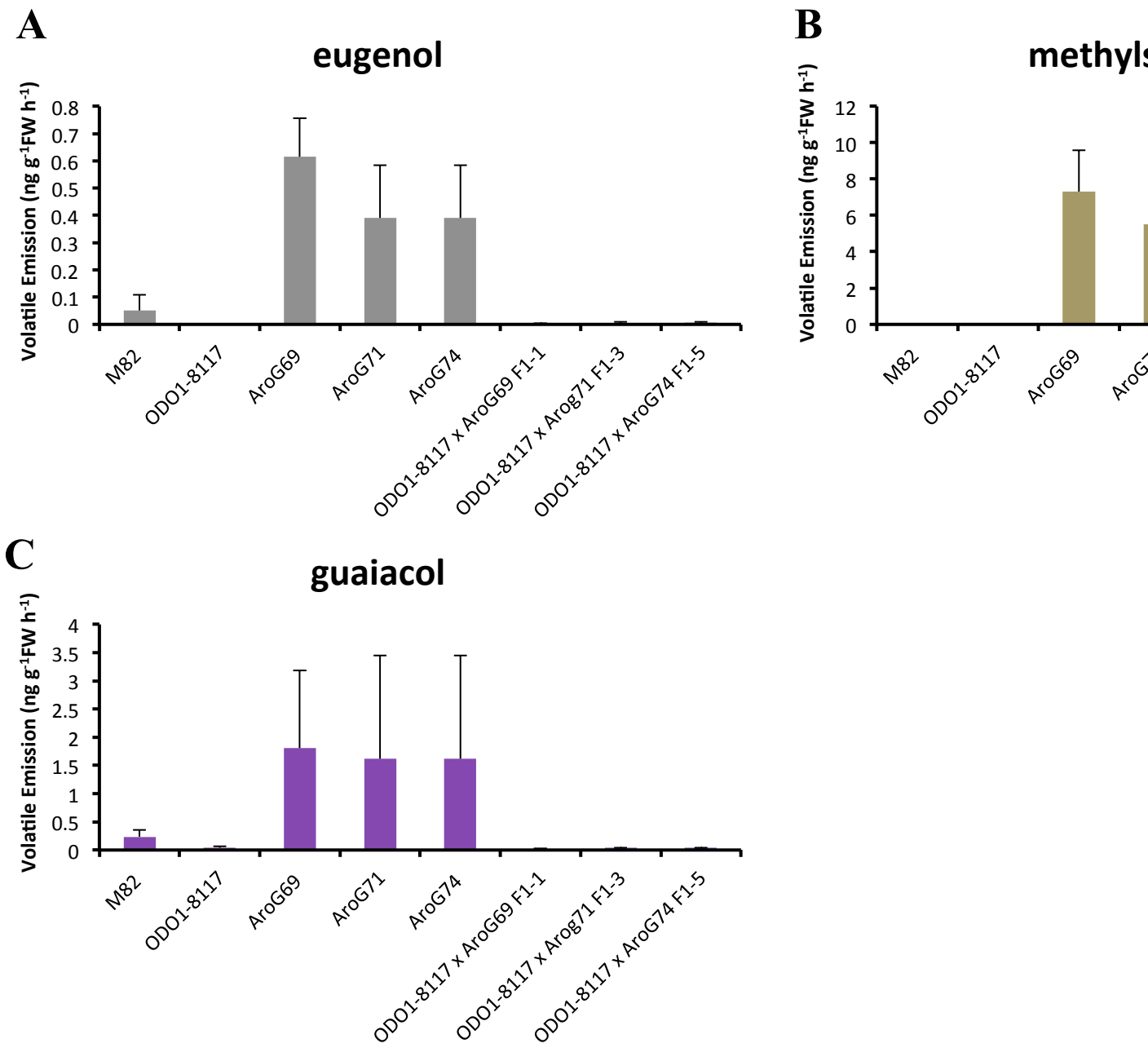
Fig. 3



Data from Klee's lab

Fig. 4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AroG71
AroG74
ODO1-8117 x AroG69 F1-1
ODO1-8117 x Arog71 F1-3
ODO1-8117 x AroG74 F1-5
B
methyls
Volatile Emission (ng g-1FW h-1)
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2
4
6
8
10
12
M82
ODO1-8117
AroG69
C
guaiacol
Volatile Emission (ng g-1FW h-1)
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1
1.5
2
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3
3.5
4
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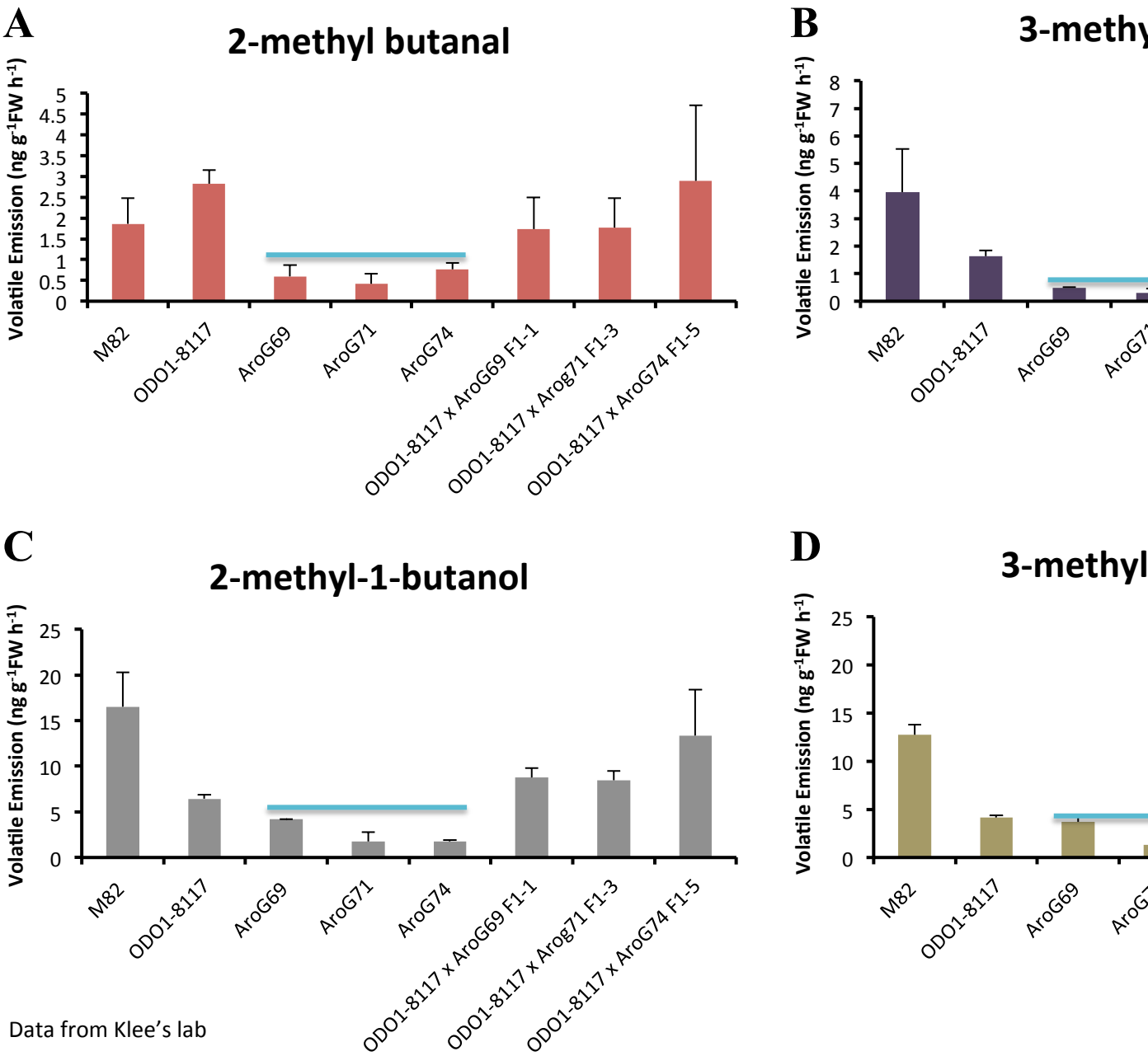
Fig. 5



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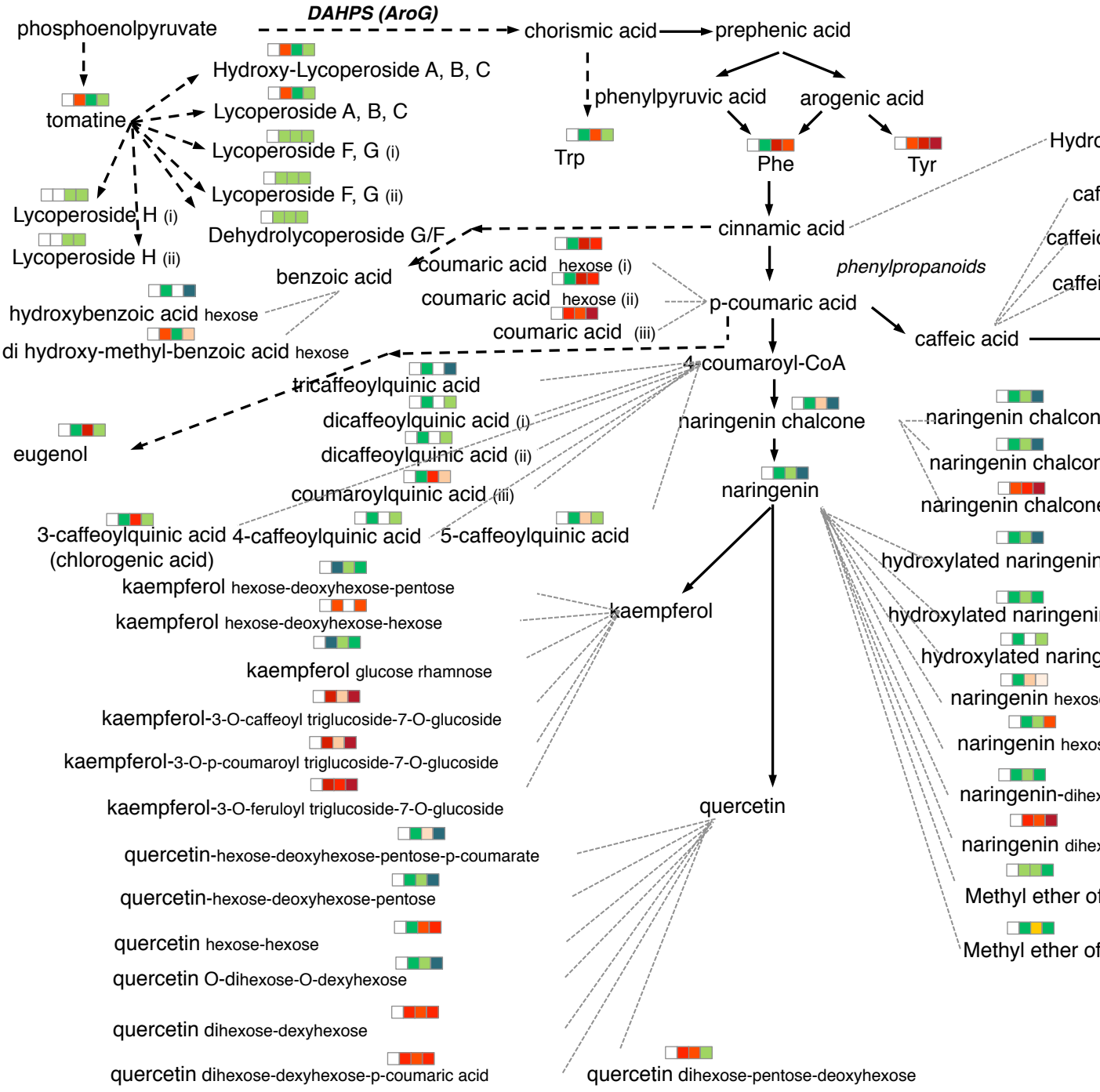
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Dehydrolycoperoside G/F
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arogenic acid
Trp
Phe
Tyr
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